A Mutated EGFR Is Sufficient to Induce Malignant Melanoma with Genetic Background-Dependent Histopathologies

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Melanoma is a tumor with a very low cure rate once metastasized. Although many genes important for melanoma induction, transformation, and metastasis have been identified, the process of melanomagenesis is only partly understood. Melanoma mediators are easiest to investigate in cell culture models, but animal models are required to evaluate their importance in the context of the whole organism. Here, we describe a transgenic melanoma model in medaka. The oncogenic receptor tyrosine kinase, Xmrk, responsible for melanoma formation in *Xiphophorus*, was stably expressed under the control of a pigment cell-specific promoter. The transgenic fish developed pigment cell tumors with a penetrance of 100%. The model was used for monitoring the *in vivo* relevance of several apoptosis and differentiation genes, and for induction of melanoma-relevant signal transduction pathways. We found that Stat5 activation, and Mitf and Bcl-2 levels correlated with a more aggressive stage of the malignancy. Interestingly, different types of pigment cell tumors occurred depending on the genetic background, namely invasive melanoma, uveal melanoma, or exophytic and less aggressive pigment cell tumors called xanthoerythrophoroma. Furthermore, on p53 mutant background, the expression of *xmrk* led to the appearance of giant focal pigment cell tumors, whereas tumor onset was unchanged compared with wild-type medaka.

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INTRODUCTION

Malignant melanoma in humans shows a steady increase in incidence and still remains one of the deadliest cancers, with a 5-year survival rate of less than 20%. A number of genes have been identified that can be ascribed a critical role during development of the disease, at least in a subset of various types of melanoma, such as *NRAS, BRAF, PTEN, CDKN2A, CTNNB1* (β-catenin), and *GNAQ* (Castellano and Parmiani, 1999; Chin, 2003; Van Raamsdonk *et al.*, 2009). To understand, in detail, the function of these genes for melanoma development and the molecular processes underlying transformation of a normal pigment cell into a fully malignant melanoma cell, animal models can make a useful contribution. Transgenic models have the advantage that the primary effects elicited by the tumor-inducing gene can be separated from the contribution of the genetic background. As efficient therapies are urgently needed, animal models can also be used for target validation and high-throughput drug screening.

One of the oldest animal models for human cancer are *Xiphophorus* fish, in which spontaneous melanoma formation occurs specifically in certain hybrid genotypes. Here, overexpression of a mutated version of the EGF receptor, called Xmrk, causes development of malignant tumors (for review see Meierjohann and Schartl, 2006). The signal transduction events downstream of Xmrk are reasonably well understood. It is, however, unclear to what extent the hybrid genetic background and potential ''tumor modifiers'' contribute to Xmrk-induced tumorigenesis. In addition, further studies, especially large-scale high-throughput approaches, are hampered by the fact that this fish is live-bearing and transgenic approaches are not available. In the medaka, no natural occurring tumor model has been found, but this species offers all advantages of small-animal experimental systems. Similar to zebrafish, it is easy to breed in large numbers, it produces externally developing transparent embryos, and available transgenic technologies provide easy means to modify the genome (Wittbrodt *et al.*, 2002; Thermes *et al.*, 2002). In addition, a number of *in vitro* systems have been established, including embryonic stem cell-like and spermatogonial stem cell lines (Hong *et al.*, 1998; Hong *et al.*, 2004). The full genome has been sequenced (Kasahara

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Abbreviations: MAPK, mitogen-activated protein kinase; Mitf, microphthalmia-associated transcription factor

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et al., 2007) and large-scale mutagenesis screens have uncovered numerous mutants with interesting phenotypes (Furutani-Seiki *et al.*, 2004). Such mutants can be exploited in search for genetic modifiers, as it was done in the zebrafish model (Pei *et al.*, 2007; Yeyati *et al.*, 2007). Furthermore, established methods, such as tilling, offer the possibility to access genetic changes that modify the observed phenotype (Sood *et al.*, 2006).

For medaka, there is a fair collection of already described pigmentation mutants available, which can complement the analogous zebrafish collection (Kelsh *et al.*, 2004; Pickart *et al.*, 2004; Lynn Lamoreux *et al.*, 2005). In both fish models, this offers the opportunity to monitor melanoma development in a pigmentation mutant context and thereby elucidate whether in addition to the pigment cell-specific transcription factor, microphthalmia-associated transcription factor (Mitf), as described for human melanoma (Du *et al.*, 2004; Carreira *et al.*, 2006; Wellbrock *et al.*, 2008), other pigment cell-specific genes also contribute to melanoma development.

We therefore aimed at using the advantages of the medaka by transferring *xmrk*-dependent melanoma development from *Xiphophorus* to this species. By using a pigment cell-specific promoter, we obtained several stable transgenic medaka lines that spontaneously develop malignant melanoma with 100% penetrance. Analysis of these lines uncovered the existence of tumor modifier genes in the medaka genome and pointed out melanoma-relevant intracellular signaling events.

RESULTS

Melanoma formation in transgenic medaka

In the *Xiphophorus* melanoma model, tumorigenic expression of *xmrk* occurs only in melanocytes. However, previous analyses have shown that *xmrk* is a potent oncogene that can also transform a variety of other cell types (Winkler *et al.*, 1994; Winnemoeller *et al.*, 2005). Owing to this, the generation of a transgenic melanoma model critically depended on a pigment cell-specific promoter to drive *xmrk* expression. Thus, the *mitfa* promoter from medaka was fused to the full-length cDNA of the *Xiphophorus xmrk* oncogene.

Embryos (strains Carbio and albino (i-3)) injected with this construct developed a number of abnormalities due to the activity of the transgene. Frequently, single abnormally large pigment cells, whose morphology resembled the typical macromelanophores of *Xiphophorus*, were noted. Whether these large pigment cells are multinucleated and arise through Xmrk-driven endomitosis, as shown in murine melanocytes (Leikam *et al.*, 2008), remains to be addressed. Importantly, this cell type does not occur in non-*xmrk*-transgenic medaka. Some embryos showed large black patches of hyperpigmentation at 5–6 days of development (Supplementary Figure S1). Other embryos developed three-dimensional growth of darkly pigmented cells, which were histologically classified as melanotic tumors (data not shown). None of these fish hatched and survived.

Some of the injected fish developed, as juvenile or adult animals, large areas of hyperpigmentation or progressively growing pigment cell tumors. Besides melanoma, pigment cell tumors of the red and yellow pigment-containing xanthophores and erythrophores (so called xanthoerythrophoromas or XE tumors) also developed (Figure 1a), sometimes even on the same individual.

The melanotic tumors showed highly invasive growth, penetrating mainly into the body musculature and also into internal organs. In some fish, the tumors started to develop from the chorioidea of the eye, from the spinal cord or from the intestine (Figure 1b–d, Supplementary Figure S2). In fish, these organs harbor extracutaneous melanocytes, apparently giving rise to the observed melanoma.

Importantly, melanoma tissue was also detected at sites, which are normally devoid of extracutaneous pigment cells, for example, the liver (Figure 1c, lower left and Supplementary Figure S2). Histological analysis from serial sections revealed that the tumors had no connection to the main tumor mass and can, therefore, be regarded as metastases.

The melanotic lesions showed different degrees of pigmentation. The more aggressive melanomas were always less melanized, with large fractions of undifferentiated cells exhibiting nuclear pleomorphisms similar to human melanoma. Both xanthoerythrophoromas and melanomas were composed of densely packed cells with pleomorphic nuclei, eventually invading the underlying musculature. Isolated nests of melanized cells were regularly seen in these neoplasms (Figure 1a, bottom).

On ultrastructural level, the presence of premelanosomes as predominant organelles confirmed the low degree of melanoma cell differentiation. Cells were also characterized by the typical irregularly shaped lobulated nuclei exhibiting nuclear pockets (Figure 2a and b). Xanthoerythrophoroma cells were typically densely packed with two types of spherical or oval cytoplasmic organelles limited by a single membrane. The smaller ones were completely filled with amorphous substance. The larger ones were either empty or contained amorphous electron-dense material loosely arranged in circular structures (Figure 2c and d). These vesicles correspond to the described ultrastructure of larval and adult-type pterinosomes that are responsible for the reddish and yellowish color (Kamei-Takeuchi and Hama, 1971; Frost *et al.*, 1984). The appearance of the observed tumor-related melanosomes or pigment-containing organelles is in sharp contrast to the well defined and pigment-loaded mature melanosomes or other pigment-containing organelles that were previously described for normal fish pigment cells (Riehl *et al.*, 1984; Ferrer *et al.*, 1999; Hirata *et al.*, 2005).

Stable transgenic lines were established from the injected G0 fish. Tumors in the stable lines showed the same spectrum, and were of the same histology and ultrastructure as the lesions in the G0 animals.

The comparison between heterozygous and homozygous *mitf*::*xmrk* transgenic fish revealed a marked dosage effect. Heterozygous fish first developed extended dark black spots in the fins and the trunk at about 6–10 weeks of age. According to Patton *et al.* (2005) and Gimenez-Conti *et al.* (2001) they were classified as f-nevi or cutaneous hyperpigmentation. Furthermore, most of these transgenic fish showed a hyperpigmentation of the xanthophore/erythrophore line-

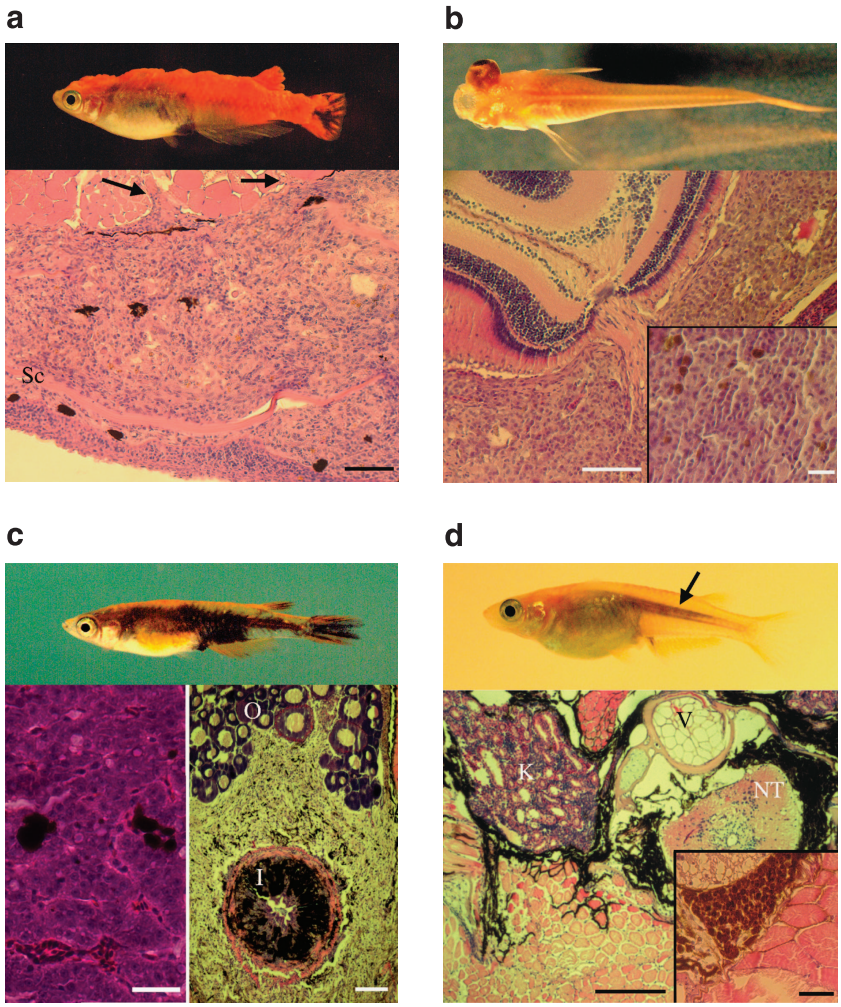

Figure 1. Histology of pigment cell tumors in *mitf*::*xmrk* transgenic medaka. (**a**) Displays of macroscopic and microscopic patterns of exophytic xanthoerythrophoroma. (**a**, upper image) 10-week-old female medaka (*Carbio*) with exophytically growing xanthoerythrophoroma. (**a**, lower image) Xanthoerythrophoroma growing in the dermal compartment and locally invading the underlying trunk musculature (arrows). Nests of melanized tumor cells are interspersed (Sc, scale). Bar = 50 µm. (**b**) Macroscopic and microscopic patterns of amelanotic uveal melanoma. (**b**, upper image) 5-month-old adult albino medaka (*i-3*) exhibiting unilateral exophthalmus and light brownish tumorous outgrowth around the eye. (**b**, lower image) Origin of the tumor in the chorioidea. The histological structures of retina and optical nerve seem to be normal and show no sign of malignancy. Bar = 50 µm. (**b**, insert) The tumor cells are of epitheloid uveal melanoma cell type, forming parallel rows. Some of them are lightly pigmented, which is a typical feature of amelanotic melanoma in tyrosinase-positive albino genotypes. Bar = 20 µm. (**c**) Macroscopic and microscopic patterns of invasive extracutaneous melanotic melanoma. (**c**, upper image) 4-week-old juvenile medaka (*Carbio*) with melanotic tumor filling the abdomen and growing invasively into the musculature. (**c**, lower left image) Melanoma metastasis in the liver. Bar = 40 µm. (**c**, lower right image) Melanoma growing in the intestinal mucosa (I), filling the body cavity and growing invasively into the ovary (O). Bar = 100 µm. (**d**) Macroscopic and microscopic patterns of perineural melanotic melanoma. (**d**, upper image) 11-week-old female medaka (*Carbio*) with melanized tissue surrounding the neural tube (arrow). (**d**, lower image) Advanced stage of perineural melanoma growing progressively into the abdomen and the dorsal trunk musculature. Kidney (K), vertebral bone with remnants of the chorda dorsalis (V), neural tube (NT). Bar = 150 µm. (**d**, insert) Early stage of perineural melanoma consisting of heavily melanized differentiated cells that have not yet invaded the adjacent muscle. Bar = 40 µm.

age, leading to intensive dark orange coloration of large areas of the skin. A varying fraction (10–40%) developed exopyhtic and/or invasive pigment tumors. This process could take up to 6 months after first appearance of hyperpigmentation.

In all homozygous fish, the pigmentation change started much earlier, sometimes as early as 8–10 days post hatching. Within 2–6 weeks, the hyperpigmented areas transformed to malignant tumors. Thus, similar to the *Xiphophorus* model, melanoma penetrance also reached 100% in the transgenic medaka model. After a few weeks, most tumor-bearing fish became weak, developed cachexia, and eventually died.

Signal transduction and gene expression of xmrk-induced tumors

All tumors analyzed showed a high mRNA expression ($n = 28$) and low to very high protein expression of the stably integrated *xmrk* transgene ($n = 10$) (exemplarily shown in Figures 3 and 4). Western blot analyses comparing the activation state of known signal transducers in *Xiphophorus* and medaka melanoma showed that Xmrk expression was accompanied by activation of Akt, consistent with the known stimulation of the Pi3 (phosphoinositide 3) kinase pathway by Xmrk (Wellbrock *et al.*, 1999). The well-established

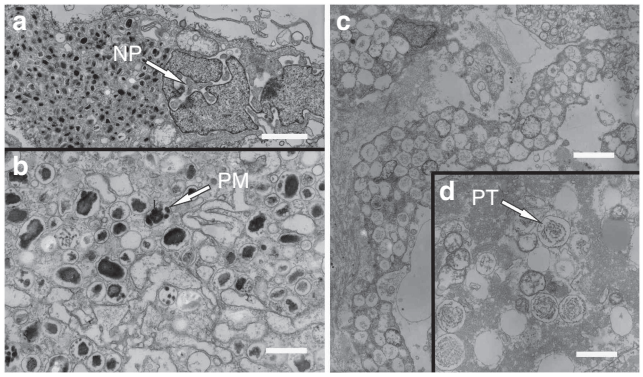

Figure 2. Ultrastructure of melanoma and xanthoerythrophoroma. (**a**) Melanoma cell with lobulated nucleus and nuclear pockets. The cytoplasm is filled with immature melanosomes. (**b**) Incompletely melanized premelanosomes with clumped or rod-like aggregates of melanin deposits. (**c**) Tumor cell from a xanthoerythrophoroma with numerous vesicular pigment organelles. (**d**) Pterinosomes of the larval type. For comparison with the ultrastructure of normal fully differentiated pigment cells see Vielkind and Vielkind (1970) and Riehl *et al.* (1984). Bars represent 4 mm in (**a**), 1 mm in (**b**), 3 mm in (**c**) and 1 mm in (**d**). NP, nuclear pocket, PM, premelanosome, PT, pterinosome.

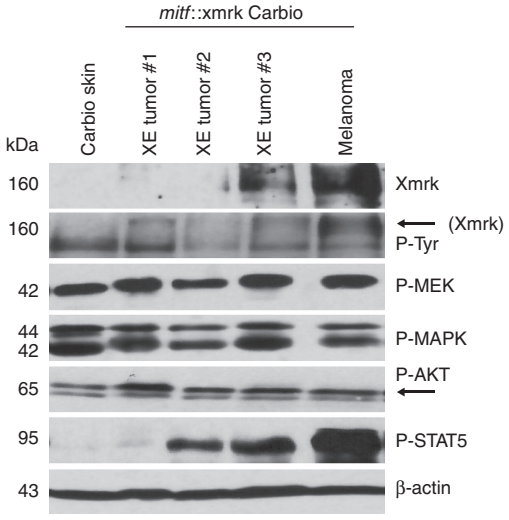

Figure 3. Western blot analysis of *Xiphophorus* hybrid and transgenic medaka tumors. Protein lysate of Carbio skin, xanthoerythrophoroma (XE tumors) and melanoma was analyzed using western blot. Antibodies were directed against Xmrk, phosphotyrosine (P-Tyr), phosphorylated MAPK/ERK kinase (P-MEK), MAPK (P-MAPK), AKT (P-AKT), and STAT5 (P-STAT5). After long exposition of the blots, an Xmrk-specific signal was also observed in XE tumors #1 and #2 (not shown). The upper P-Tyr band (arrow) runs at the same size as the Xmrk-specific band, suggesting that it represents phosphorylated Xmrk. The upper AKT band is the specific one and is indicated by an arrow. β-actin is used as loading control. Total levels of STAT5 were similar between skin and pigment cell tumors from each species (data not shown).

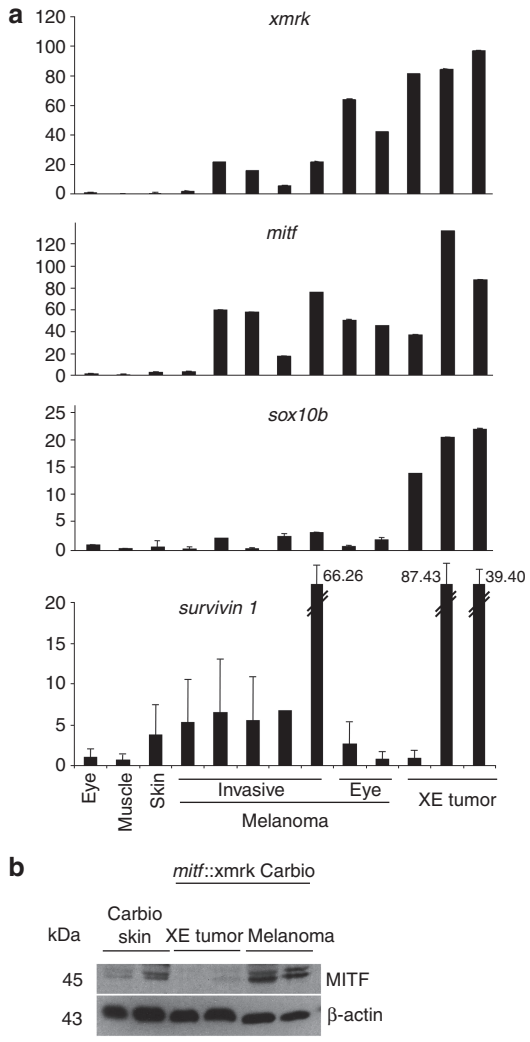

Figure 4. Expression of *xmrk, mitf, sox10b,* and *survivin 1* in tumors. (**a**) Quantitative real-time RT-PCR analysis of *xmrk, mitf, sox10b, and survivin 1* transcript levels in normal eye, muscle, skin, and in different pigment cell tumor types of *mitf*::*xmrk* transgenic medaka. (**b**) Western blot of MITF in control skin of healthy Carbio fish, xanthoerythrophoroma (XE tumor), and melanoma. β-actin was used as loading control.

activation of Mapk/Erk kinase and mitogen-activated protein kinase (Mapk) by Xmrk (Wellbrock *et al.*, 2002) was not so evident in the transgenic model, as healthy medaka skin, used as reference organ, already exhibits a considerable activity of both signal transducers. Most striking was the strong activation of Stat5 that went along with the level of Xmrk expression in the tumors.

The fact that Xmrk signaling is the primary event inducing pigment cell tumors in this transgenic model prompted us to analyze whether genes, known to be relevant for human melanomagenesis, are affected by Xmrk. In the tumors, no significant transcriptional differences were recorded for the growth inhibitory gene, *cdkn2d*, or the growth-promoting genes, *stat3*, *stat5*, and *b-raf* (data not shown). In contrast, there was a strong transcriptional upregulation of the pigment cell-specific transcription factor *mitf* that went along with *xmrk* expression (Figure 4a). Although melanoma and xanthoerythrophoroma both displayed this upregulation on RNA level, enhanced Mitf protein levels were only observed in melanoma, whereas xanthoerythrophoroma had undetectable to low protein levels (Figure 4b). We also monitored

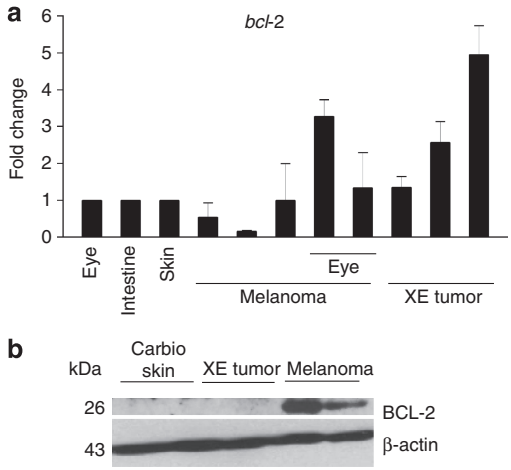

Figure 5. Expression of *bcl-2* in tumors. (**a**) Quantitative real-time RT-PCR analysis of *bcl-2* transcript levels in normal eye, intestine, skin, and in different pigment cell tumor types of *mitf*:: *xmrk* transgenic medaka. *Ef1a* was used as reference gene. (**b**) Western blot of BCl-2 in control skin of healthy Carbio fish, xanthoerythrophoroma (XE tumor), and melanoma. β-actin was used as loading control.

another transcription factor playing an important role in pigment cell development, *sox10*, which can induce *mitf* expression in zebrafish. Surprisingly, it was highly upregulated in all exophytic xanthoerythrophoroma, but only slightly in some uveal tumors and invasive extracutaneous melanoma (Figure 4a). Thus, it did not correlate with Mitf protein expression. This leads to the conclusion that Mitf is regulated by posttranscriptional mechanisms in this melanoma model.

The antiapoptotic factor, BCL-2 plays an important role in human melanomagenesis and is considered as a target for the treatment of malignant melanoma (Tarhini and Kirkwood, 2007; Poeck *et al.*, 2008). In some melanoma and xanthoerythrophoroma from *mitf*:: *xmrk* medaka, an upregulation of medaka *bcl-2* on RNA level was visible (Figure 5a). However, protein levels were only significantly raised in melanoma tissue (Figure 5b).

We also monitored expression of several progression markers described for human melanoma, namely integrin α_v, N-cadherin, cyclin D1, telomerase, and survivin (Hodi, 2006). When comparing healthy tissue with pigment cell tumors, the only consistently upregulated gene in melanoma (except uveal melanoma) was *survivin 1*, one of the two survivin genes occurring in fish (Figure 4a).

Evidence for genetic modifiers

Most *xmrk* transgenic lines were established using the Carbio strain of medaka, which is a non-inbred line and possesses a mixed genetic background. Here, various types of pigment cell tumors were observed, with xanthoerythrophoromas displaying the highest frequencies, followed by extracutaneous melanoma, and then by uveal melanoma that only occurred rarely. We have started to cross the transgene into different medaka lines of defined genetic background. Already after two generations, we observed strong differences

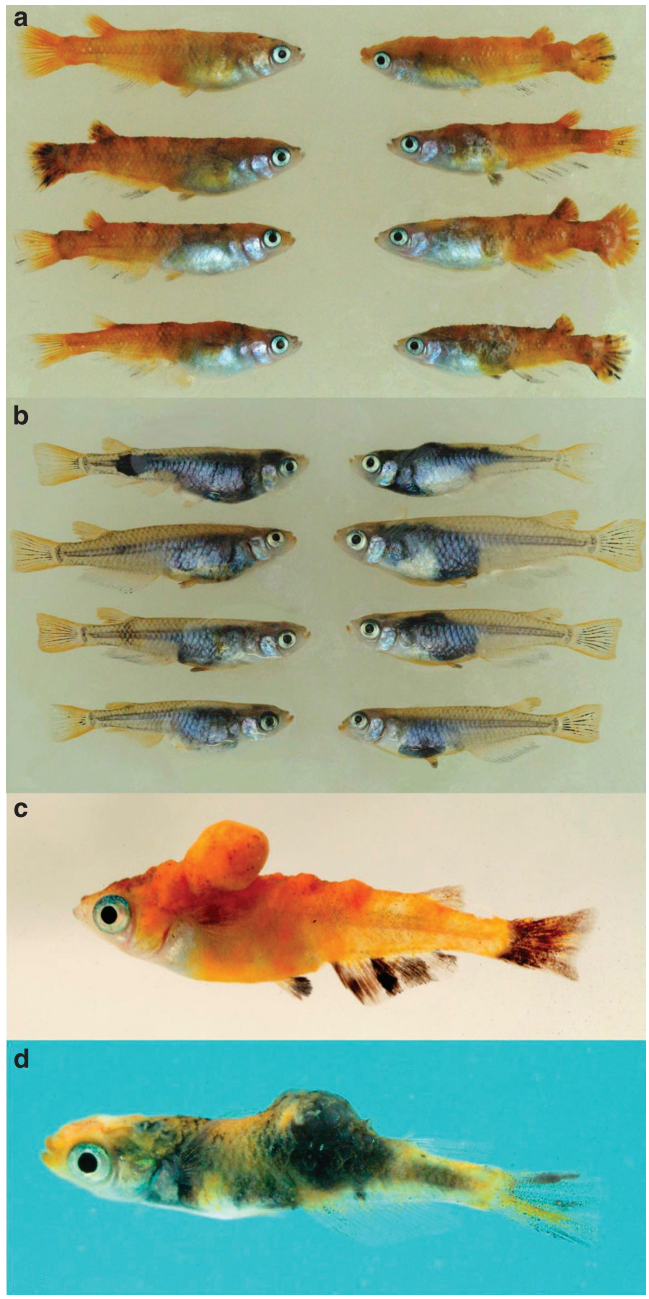

Figure 6. *Mitf*:: *xmrk* transgenic medaka showing genetic background-dependent differences in tumor development. (**a**) Cutaneous exophytic xanthoerythrophoroma in *CabR′* strain. (**b**) Extracutaneous invasive melanotic melanomas in *HB32C* strain. (**c**, **d**) *Mitf*:: *xmrk* transgenic *p53*$^{-/-}$ medaka with typical focal development of fast growing nodular tumors. (**c**) Xanthoerythrophoroma and (**d**) melanotic melanoma.

in the tumor phenotypes depending on the genetic background. In the *CabR′* and *HB32C* background, the penetrance was 100% in homozygous transgenics, similar to *Carbio*. Within each line the tumor phenotype was stereotyped with respect to tumor onset, pathophysiological growth characteristics, and location (Figure 6a). *CabR′* fish containing the *xmrk* transgene developed almost exclusively massive exophytic xanthoerythrophoromas. Only at terminal stages

these displayed different degrees of invasiveness into the underlying trunk musculature. About 2–5% of the fish had nodular or invasive melanoma and these mostly occurred in addition to the xanthoerythrophoroma.

In the *HB32C* genetic background, on the contrary, the absolute majority of tumors were classified as invasive extracutaneous melanoma, with an onset at 4–6 weeks that occasionally progressed to large nodules breaking through the integument. Xanthoerythrophoroma were extremely rare in this strain (Figure 6b).

In the albino (*i-3*) genetic background, the *xmrk* carrying fish showed a hyperpigmentation of xanthophores that rarely developed into tumors. About 30% developed extracutaneous, weakly pigmented melanoma from the intestine. Strikingly, up to 40% of this strain exhibit unilateral or bilateral eye melanoma. By histology, these tumors were shown to emerge from the chorioidea and not from the retinal pigment epithelium (Figure 1c).

The *mitf*::*xmrk* transgene was also crossed into a *p53*-negative genetic background (Taniguchi *et al.*, 2006). Neither heterozygous nor homozygous *p53*-mutant fish displayed, during the initial phase of tumorigenesis, a marked difference in tumor onset or any other pathological feature compared with the *p53* wild-type *Carbio* background. However, when the fish reached adulthood, all homozygous $p53^{-/-}$ fish developed one to several rapidly growing exophytic or invasive nodular tumors that reached a strikingly large size (Figure 6c and d). These tumors most likely arose from already existing melanoma or xanthoerythrophoroma, and did not develop *de novo* from non-tumorous tissues. So far we did not observe this phenomenon in the $p53^{+/-}$ *mitf*::*xmrk* fish after more than 1 year of observation.

DISCUSSION

The *xmrk* gene is a highly potent melanoma-inducing oncogene derived from the *Xiphophorus* EGFR gene, which leads to neoplastic transformation of a variety of cell types of fish and mammalian origin. EGFR and related growth factors are expressed in normal human melanocytes, and stimulation with ligand promotes their migration (Gordon-Thomson *et al.*, 2001; Stove *et al.*, 2003; Gordon-Thomson *et al.*, 2005). They are also expressed in melanoma cells, and are associated with melanoma proliferation *in vitro* (Stove *et al.*, 2003; Gordon-Thomson *et al.*, 2005; Funes *et al.*, 2006) and with melanoma maintenance *in vivo* (Bardeesy *et al.*, 2005). Here, we show that expressing *xmrk* under the control of a pigment cell-specific promoter is sufficient to induce melanoma in medaka. Similar to human pigment cells, but unlike murine ones, fish pigment cells mainly reside in the basal layer of the epidermis. All cutaneous melanoma originate from there. The *xmrk*-induced melanoma observed here show striking ultrastructural and histopathological similarities to human melanoma. As in humans, the fish melanoma cells contain strongly deformed nuclei and immature melanosomes, which are characteristic for poorly differentiated melanoma. Metastases to the liver are also often observed (Vielkind and Vielkind, 1970; Riehl *et al.*, 1984; Gimenez-Conti *et al.*, 2001). Thus, this first stable transgenic fish melanoma model provides an useful additional tool for studies on genetic and biochemical requirements for this cancer type. Importantly, we also established strains that develop uveal and extracutaneous melanoma, depending on their genetic background. Uveal melanomas are also observed in human patients. The mostly used murine uveal melanoma model relies on injection of uveal melanoma cells of murine or human origin into the eye, thus allowing to monitor maintenance and progression, but not initial development of the tumor (Notting *et al.*, 2006; Yang *et al.*, 2008). In addition, there is a transgenic murine model for pigmented ocular neoplasm, in which large T antigen, driven by the *Tyrp* promoter, is causative for the disease (De la Houssaye *et al.*, 2008). However, owing to the induction of known melanoma-relevant pathways and its full passage through all steps necessary for development of real uveal melanoma, the medaka model is the first tumor model that carries the potential to recapitulate this specific melanoma subtype. The presence of different pigment cell tumor phenotypes in our model system indicates a genotype-dependent action of tumor modifier genes and will allow their identification using genetic and large-scale mutagenesis approaches. Remarkably, this previously unknown melanoma model also shows a pronounced metastatic phenotype, which is less evident in the original *Xiphophorus* model. The possibility of influencing pathological features by differences in the genetic background combined with the availability of genomic tools and transgenic technology opens previously unreported opportunities for an in-depth analysis of the molecular mechanisms of melanoma development that were not available before.

The new melanoma model was used to evaluate *in vivo* pigment cell tumor-relevant molecules. We found that activation levels of Stat5 strictly correlated with Xmrk expression levels in both xanthoerythrophoroma and melanoma, the latter displaying the highest activation. In comparison, Mek, Mapk, and Akt also occurred in their activated forms in non-melanoma fish skin, although usually in lower levels. It is possible that the slight increase of activated Akt in melanoma is sufficient to induce aberrant growth signaling and melanoma progression, as it might overcome a certain critical threshold level. In addition, the Mek/Mapk and Pi3K/Akt pathways might be important in early stages of melanoma formation, and their activation might be less prominent in advanced melanoma that were used for western blot analysis.

However, Stat5 seems to play a dominant role, at least in already developed pigment cell tumors. The importance of Stat5 for Xmrk-driven melanoma in *Xiphophorus* was inferred from cell culture studies, as it takes part in *xmrk*-transformed mouse melanocytes in antiapoptotic and proliferative signalling (Wellbrock *et al.*, 1998; Baudler *et al.*, 1999; Morcinek *et al.*, 2002). In addition, in human melanoma cell lines, STAT5 was shown to be involved in both survival and interferon resistance (Wellbrock *et al.*, 2005; Mirmohammadsadegh *et al.*, 2006).

An upregulation of Mitf protein was only observed in Xmrk-induced melanoma compared with that in healthy skin.

In xanthoerythrophoroma, Mitf levels were strongly decreased. The role of this transcription factor in melanoma development is not entirely understood. On the one hand, it seems to be an antiproliferative factor in general (Carreira *et al.*, 2005) and in melanoma cells with upregulated RAS/RAF/MAPK signalling. However, a certain threshold level needs to be retained to stimulate proliferation and/or survival (Carreira *et al.*, 2006; Wellbrock and Marais, 2005). On the other hand, high-level amplification of the *MITF* gene in metastatic melanoma correlated with poor prognosis. *In vitro* studies showed a cooperative effect of MITF with mutant BRAF in the transformation of primary melanocytes (Garraway *et al.*, 2005). $BRAF^{V600E}$ can also enhance *MITF* transcription in melanoma, thus effecting expression of the crucial cell cycle kinases, *CDK2* and *CDK4* (Wellbrock *et al.*, 2008). In addition, upregulation of Mitf protein could have a tumor promoting effect, as in humans, the antiapoptotic factor *BCL*-2 is a direct MITF target gene (McGill *et al.*, 2002). Our *in vivo* data support the previous *in vitro*-based hypothesis that Mitf might play a pro-tumorigenic role—at least in the more malignant melanoma.

When we monitored Bcl-2 in medaka pigment cell tumors, we found a correlation between Mitf and Bcl-2 protein, which was aberrantly present in melanoma, but not in xanthoerythrophoroma. Both are more strongly regulated by posttranscriptional than by transcriptional mechanisms in medaka tumors. McGill *et al.* (2002) discussed a specific role for BCL-2 in the protection against chemical stress that is associated with melanin synthesis, which could explain the observed differences between melanoma and xanthoerythrophoroma. A possible involvement of Mitf or Bcl-2 in the more malignant phenotype of the melanoma can now be addressed in future studies.

The comparable activation levels of MAPK in melanoma and medaka skin, and the high mRNA level of *mitf* that is not reflected by a similar increase in protein show that high MAPK activity and *mitf* expression levels may be useful markers for melanoma, but are not necessarily similarly important on the functional level for the development of the tumor.

Another interesting observation was the high expression of *survivin 1* in one invasive melanoma and in XE tumors, but not in healthy tissue. Owing to the lack of a fish-specific survivin antibody, it was not possible to examine protein expression. However, we noted that only one of the two survivin genes was regulated. An involvement of survivin 1 in angiogenesis was shown during zebrafish development (Ma *et al.*, 2007). In human melanoma, survivin is often strongly expressed, whereas it is downregulated in normal human melanocytes by a mechanism involving p53, Rb, and E2F2 (Raj *et al.*, 2008). It has a dual function in either preventing apoptosis (when localized to mitochondria) or mediating cell cycle progression (when associated with the chromosomal passenger complex). Most likely, both tasks are fulfilled in melanoma (Ding *et al.*, 2006; Liu *et al.*, 2006). In melanoma patients, survivin expression is even associated with disease recurrence and poor survival (Piras *et al.*, 2007).

A melanoma model for zebrafish was published earlier by Patton *et al.* (2005). Here, melanomas were produced by transgenic expression of human $BRAF^{V600E}$ under control of the homologous *mitf/nacre* promoter. The V600E mutant is frequently found in human melanoma and nevi. It leads to a constitutive hyperactive kinase activity (Wan *et al.*, 2004) and consequently to a high MAP kinase activity, which is a hallmark of many melanomas (Satyamoorthy *et al.*, 2003). The oncogenic *BRAF* mutation is comparable with the situation following growth factor receptor signaling, including the EGFR and Xmrk. However, unlike the *xmrk* transgenic medaka, expression of activated $BRAF^{V600E}$ alone in zebrafish resulted in benign hyperpigmentation spots, called f-nevi. Only when mutated *BRAF* was highly overexpressed in pigment cells of p53-deficient zebrafish (Berghmans *et al.*, 2005), malignant melanoma developed. The zebrafish data support the observation that BRAF activation in humans is not sufficient for melanoma formation (Michaloglou *et al.*, 2005), but presumably induces a senescence-like cell cycle arrest when present in nevi (Pollock *et al.*, 2003).

A common feature of the zebrafish and medaka models is that in both cases a p53 effect on tumor development is noted. However, in the transgenic medaka, absence of a functional p53 protein had no effect on the onset of melanoma formation and the degree of malignancy during early phases of the disease. Instead, the absence of p53 promoted the development of massive focal outgrowth or invasion, indicating its importance for melanoma progression. Thus, in medaka, the absence of dysfunctional p53 is not a precondition for malignant melanoma development in general. The role of p53 deficiency for melanoma needs to be elucidated further in both models, as p53 itself is rarely mutated in human melanoma development (for review see Levy *et al.*, 2006; Takata and Saida, 2006).

Xmrk as a receptor tyrosine kinase is strategically placed at the top of several growth-promoting signaling pathways. It has been shown to activate, beside the RAS/RAF/MAPK cascade, the PI3 kinase, PLC-gamma, STAT5, FYN, and the focal adhesion kinase, all of which are involved in mediating the complex fully malignant neoplastic phenotype (Wajapeyee *et al.*, 2008). Thus, the stronger oncogenicity of Xmrk compared with that of $BRAF^{V600E}$ may be simply explained by its wider downstream activation capacity.

We report here the first stable transgenic melanoma model in a small aquarium fish species, in which all gene carriers develop a tumor. The stable *xmrk* transgenic lines give a very stereotyped tumor development with an early onset during larval stages. They are, therefore, ideally suited for large-scale testing of chemical compound library and for mutagenesis screens to identify tumor modifier genes.

MATERIALS AND METHODS

Fish

All animal studies have been approved by the authors' Institutional Review Board (Animal Welfare Officer of the University of Wurzburg). Adult fish were maintained under standard conditions (Kirchen and West, 1976) with an artificial photoperiod (10 hours of darkness, 14 hours of light) to induce reproductive activity. Clusters of fertilized eggs were collected 0.5–1 hour after the onset of light and kept in a rearing medium containing 0.1% NaCl, 0.003% KCl,

0.004% $CaCl_2 \times 2H_2O$, 0.016% $MgSO_4 \times 7H_2O$, and 0.0001% methylene blue. Embryos were staged according to Iwamatsu (2004).

Fish from the following strains were used: *Carbio* (outbred strain, mixed genetic background of southern medaka), *CabR′* (closed colony inbred strain carrying the *R′*, *variegated*, locus), albino *i-3*, *HB32C*. (For detailed information on these strains see http://www.shigen.nig.ac.jp/medaka). In addition, the $p53^{-/-}$ strain was used, which owing to a point mutation has a premature stop codon in the p53 gene (E241X) and consequently lacks expression of a functional p53 protein (Taniguchi *et al.*, 2006). The $p53^{-/-}$ tilling mutation is kept in the *CabR′* genetic background.

Isolation of the medaka mitf promoter and construction of expression vectors

To isolate a fragment of the medaka *mitfa* promoter, a PCR was performed using genomic DNA of the Carbio strain as template. The primers used for amplification of 1057 bases upstream of the *mitfa* gene introduced an *Apa*I site and an *Nco*I site. The product was cut with *Apa*I/*Nco*I and cloned into the *Apa*I/*Nco*I-digested 3.9 kbp fragment of plasmid *I-Sce-I-pCSKA*::*deltaDM*::*GFP* (Hornung *et al.*, 2007), containing the enhanced green fluorescent protein open reading frame with the SV40 polyA site. This resulted in plasmid *mitf*::*GFP*. Injection of this plasmid into medaka embryos confirmed pigment cell-specific expression of transgenes under the control of this promoter (data not shown). For constructing the *mitf*::*xmrk* plasmid, the enhanced green fluorescent protein open reading frame was excised from *mitf*::*GFP* by digestion with *Nco*I/*Bsr*6I. The 3.6 kb *Xba*I/*Dra*I cDNA fragment from *xmrk* was excised from pBIIG4 (Malitschek *et al.*, 1994) and inserted into the remaining vector containing the *mitf* promoter and the SV40 polyA site. Constructs used for this study were verified by sequencing.

Production of stable transgenic medaka

For the generation of stable transgenic lines, the meganuclease protocol (Thermes *et al.*, 2002) was used. Briefly, approximately 15–20 pg of total vector DNA in a volume of 500 pl injection solution containing I-*Sce*I meganuclease was injected into the cytoplasm of one-cell stage medaka embryos (strains: Carbio, *CabR′*, *i-3*, *HB32C*, $p53^{-/-}$). Adult F0 fish were mated with each other and the offspring was tested for the presence of the transgene by PCR from pooled hatchlings. Siblings from positive F1 fish were raised to adulthood and tested by PCR from dorsal fin clips, as described (Altschmied *et al.*, 1997).

Altogether, 1,139 *Carbio* and 801 albino (*i-3*) embryos were injected with the construct, giving rise to 105 (*Carbio*) and 51 (*i-3*) adult individuals. Eight (*Carbio*) and three (*i-3*) of these were carriers of the transgene. Stable lines were established, three of which (two *Carbio* and one *i-3*) showed a pigmentation phenotype.

Histology

For histological analysis, fish were fixed in Bouin's fixative (saturated picric acid in H_2O: formaldehyde (37%): glacial acidic acid, 15:5:1), transferred to fixative solution (1 × phosphate-buffered saline, 10% formaldehyde (3%)), and embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin/eosin.

For electron microscopy, pieces of tumor tissues were washed with phosphate-buffered saline and fixed (2.5% glutaraldehyde, 50 mM cacodylate (pH 7.2), 50 mM KCl, and 2.5 mM $MgCl_2$) overnight at 4°C. Thereafter, they were washed five times with 50 mM cacodylate buffer (pH 7.2) and post-fixed for 3–4 hours with 2% OsO_4 in 50 mM cacodylate (pH 7.2) buffer. After five washing steps with H_2O, embryos were stained with 2% uranylacetate overnight and washed again with H_2O. After gradual dehydration with ethanol, they were transferred to propylenoxid and subsequently embedded in Epon 812 (Serva, Heidelberg, Germany). Sections were analyzed using an EM10 from Zeiss (Wetzlar, Germany).

RNA expression analysis

Total RNA was extracted from pooled healthy skin or organs, as well as individual tumors using TRIZOL (Invitrogen, Karlsruhe, Germany) or Total RNA Isolation Reagent (ABgene, Hamburg, Germany) according to the supplier's recommendation. After DNase treatment, reverse transcription was performed from total RNA using Superscript II Reverse Transcriptase (Invitrogen) or RevertAid First Strand Synthesis kit (Fermentas, St Leon-Rot, Germany) and random hexamer primers, according to the manufacturer's instructions. cDNA from 15 ng of total RNA for *ef1a* and from 50 ng of total RNA for all other transcripts was used for real-time PCR (primer sequences, are made available on request) using SYBR Green reagent, and amplification was monitored with i-Cycler (Bio-Rad, Munich, Germany). All results are averages of at least two independent reverse transcription reactions and 2–5 PCR experiments from each such reaction. For quantification, data were analyzed by the delta C_t method (Simpson *et al.*, 2000) and normalized to the housekeeping gene *ef1a* mRNA.

For spot check, negative control RNA (not reversely transcribed), was used in the PCR reaction. Data are presented as mean ± standard deviation. Changes in mRNA expression were tested using an independent *t*-test with significance level $P \leqslant 0.05$.

Protein analysis

After dissection, melanoma or control tissue was rinsed with phosphate-buffered saline and homogenized in protein lysis buffer (50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.5), 150 mM NaCl, 1,5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 200 μM Na_3VO_4, 1 mM PMSF (phenylmethanesulfonylfluoride), and 100 mM NaF). A total of 50 μg of protein lysate was separated by SDS/PAGE and transferred to nitrocellulose according to standard western blotting protocols. Primary antibodies against the following proteins were used: phosphotyrosine P-Tyr-100, phospho-ERK1/2 Tyr202/204, phospho-MAPK/ERK kinase1/2 Ser217/221, phospho-AKT Thr308, phospho-STAT5 Tyr694 (all from Cell Signaling Technology, New England Biolabs, Frankfurt, Germany), beta-actin (C4), BCL-2 (C21) (Santa Cruz Biotechnology, Heidelberg, Germany), and MITF (Aviva Systems Biology, San Diego, CA). Anti-mrk is a rabbit polyclonal antiserum obtained by immunization with a polypeptide representing a C-terminal part of Xmrk (''pep-mrk'') (Malitschek *et al.*, 1994).

As secondary antibodies, horseradish peroxidase-coupled anti-mouse (Pierce, Rockford, IL), anti-rabbit (Bio-Rad), or anti-goat (Abcam, Cambridge, UK) IgG were used.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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